A STUDY OF THE NUTRITION OF *PSEUDOMONAS PUTREFACIENS*

BY

GEORGE WILMER REINBOLD

B.S., Pennsylvania State College, 1942
M.S., University of Illinois, 1947

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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IN THE GRADUATE COLLEGE OF THE
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A STUDY OF THE NUTRITION OF PSEUDOMONAS PUTREFACIENS

Pseudomonas putrefaciens, a rod shaped, pigmented organism is considered to be the important cause of the putrid defect of stored butter. This organism is difficult to isolate from such butter as its nutritional requirements are not understood and completely satisfactory media for its isolation and cultivation are not available. To aid in meeting this need the presently reported nutritional investigation of the organism was undertaken. Two specific objectives served as the basis for all experimentation: 1. to determine the growth response of *Ps. putrefaciens* to different classes of organic and inorganic compounds. 2. To determine which of these compounds could be utilized to develop a plating medium capable of supporting maximum growth of this organism.

To this end, studies were made of the growth response of 10 different cultures of *Ps. putrefaciens* to the following compounds:

1. Nitrogenous compounds:
   a. 18 common amino acids
   b. 40 other nitrogenous compounds
2. Nonnitrogenous carbon compounds:
   a. 16 aliphatic monohydric alcohols
   b. 55 organic acids and related compounds
   c. 27 carbohydrates and closely allied substances
3. 9 B-complex vitamins

The lipolysis of butterfat by *Ps. putrefaciens* was also studied.

After these experiments had been completed, combinations of the most readily utilized substances were made, and the combination best supporting the growth of the organism

was determined. This nutritive substrate was compared with Long's Agar which is, at the present time, the best known medium for the isolation and cultivation of the organism. In this comparison, initiation of growth, luxuriance of growth, and colony pigmentation were considered as criteria of response.

Experimental Cultures

Source

Ten experimental cultures were used throughout this study. Three cultures were obtained from the American Type Culture Collection (Hammer strains 95, 180, and 184), and the seven remaining cultures were isolated from local surface waters. One additional culture, isolated from local surface water, was employed only in the study of the utilization of various organic acids.

Isolation and Identification of Cultures

Water samples suspected of containing *Ps. putrefaciens* were streaked and plated on Long's Agar. These plates were incubated at 21°C. for 5 days. Brown to reddish-brown or pink bacterial colonies again were streaked on Long's Agar and later picked and placed in litmus milk. Those tubes of litmus milk showing rapid reduction and production of a distinctly putrid odor were selected as possible cultures of *Ps. putrefaciens*. The process was repeated with these cultures and the characteristic organisms were tested for the Gram reaction, for cell morphology, for phosphatase production, and for the ability to produce the putrid defect in experimental churnings of butter. All cultures used gave the characteristic response of *Ps. putrefaciens* to these tests.

Preparation of Inocula

The cultures were maintained by weekly transfers into fresh litmus milk. To prepare the inocula, the cultures were streaked on slants of Long's Agar and then were incubated for 15 to 20 hours. The resultant growth was washed from

the slants with a standard volume of sterile distilled water and was concentrated as packed cells by aseptic centrifugation. The supernatant liquid was decanted and the cells resuspended in the original volume of sterile water. The suspension was diluted until 1 drop in 10 ml. of the various nutritive substrates to be studied showed no immediate change in optical density. Since the inocula used did not immediately influence the optical density of the many substrates, it was possible to employ uninoculated tubes of the substrates as controls to establish the zero point for growth. Unless stated otherwise, inocula prepared in this manner were used in all experiments.

Methods Of Measurement Of Growth

Several different techniques were used to study the growth response of *Ps. putrefaciens* to various nutrient compounds. These techniques were (1) the plating method, (2) turbidity measurements, and (3) agar plate auxanographic tests. The pH was adjusted in all cases to 7.0 ± 0.2. Since preliminary investigation indicated the organism grew only very meagerly in Koser's Citrate Medium, and since Koser's Citrate Medium when used normal strength as the diluent avoided the toxic effect of distilled water upon the organism, the constituents of this solution were used as the basal ingredients where possible for the formulation of all media to be studied. Koser's Citrate Medium contains 0.15 per cent sodium ammonium phosphate·4 H_2O, 0.02 per cent magnesium sulfate, 0.3 per cent sodium citrate·$2H_2O$, and 0.1 per cent monopotassium phosphate.

The method of plating and the materials employed with the exception of the diluent, the plating media, and the incubation time and temperature, conformed to the specifications set forth in the Standard Methods for the Examination of Dairy Products (1948). The plating media were Long's Agar and the special combination of nutrient compounds. All plates were incubated for 3 days at 21°C.

Turbidity measurements were made in a Lumetron photo-

electric colorimeter, Model 400, and recorded as optical density.

The growth response of 11 cultures of the organism to organic acids was measured by agar plate auxanographic tests. It was necessary to use the calcium salt of each organic acid in order to maintain the pH at the desired level. The free acid in a concentration of 0.5 per cent was added to a solid basal medium essentially free of utilizable carbon and containing $CaCO_3$. The basal medium also differed from Koser's Citrate Medium in that it contained no sodium citrate and that dipotassium phosphate·$3H_2O$ was substituted for the monopotassium phosphate. Glutamic acid was added as a standard source of organic nitrogen. The inocula were spotted on the surface of the plates and the resulting growth was compared with a negative control plate.

Statistical Analyses Of The Data

The significance of the variation within the data was determined according to methods presented by Snedecor (1946).

Discussion And Results Arranged By Substrates Studied

1. Nitrogenous Compounds

a. Amino Acids. The growth response of 10 cultures to 18 common amino acids was determined under three different conditions. These were (1) amino acids present in concentrations as found in normal whole milk, (2) amino acids added in 0.0004M concentrations, and (3) amino acids added in 0.0004M concentrations but lacking the inorganic nitrogen present in the sodium ammonium phosphate of the basal medium and containing disodium hydrogen phosphate·$12H_2O$.

Ten ml. amounts of the test solutions in optically standardized 18 x 150 mm. test tubes were inoculated and then were incubated for 14 days at 21°C. Following incubation, growth changes measured in terms of optical density were made in a Lumetron photoelectric colorimeter.

To obviate possible carry-over effects 1 drop of the first 14 day incubated culture was inoculated into another 10 ml. tube which was incubated for 14 days and another recording of growth then was made. This process was repeated to obtain a third reading.

Results — The amino acids used by most of the 10 cultures were glutamic acid, aspartic acid, isoleucine, leucine, and tyrosine. There was a distinct difference in utilization of amino acids between cultures — one culture, in particular, was capable of utilizing all of the 18 amino acids.

b. Other Nitrogenous Compounds. Forty other nitrogenous compounds, ranging from ammonium salts to natural and hydrolyzed proteins were studied. These compounds were present in a concentration of 0.1 per cent. The same technique of measuring growth and subculturing was utilized; however, an incubation period of only 5 days at 21°C. was used. In this experiment the inorganic source of nitrogen was omitted from the basal medium.

Results — As before, there were distinct differences between cultures and between nitrogen sources. The most readily utilized compounds were protolysate (Mead Johnson), casamino acids (Difco), and casitone (Difco).

2. Nonnitrogenous Carbon Compounds

a. Aliphatic Monohydric Alcohols. The growth response of 10 cultures to 16 alcohols was studied. These alcohols were methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl, isobutyl, amyl, *sec*-amyl, *tert*-amyl, isoamyl, *n*-hexyl, *pri-n*-octyl, allyl, and benzyl alcohol. The alcohols were added in a concentration of 0.1 ml. to 9.9 ml. of basal medium containing glutamic acid as a standard source of nitrogen and without the sodium citrate. The basal medium did not support visible growth as determined in turbidimetric studies and as confirmed by plating a 0.1 ml. portion of the second subtransfer after 14 days' incubation.

Results — None of the 16 aliphatic monohydric alcohols studied were utilized as a source of carbon.

b. Organic Acids and Related Compounds. By the use of the agar plate auxanographic test described previously, the growth response of 11 cultures to 55 organic acids and certain miscellaneous compounds was studied.

Results — The organic acids most readily used were propionic, butyric pelargonic, lauric, lactic, succinic, sebacic, fumaric, l-malic, α-bromo-propionic, glyceric, and gluconic acid. Of these, l-malic, fumaric, and lactic acid were foremost in promoting growth and pigmentation.

c. Carbohydrates and Allied Substances. Twenty-seven different carbohydrates and polyhydric alcohols were studied. These carbohydrates, in a concentration of 0.1 per cent, were combined with the liquid, carbon-free basal medium. The basal medium was the same as that employed in the alcohol experiment. Turbidmetric measurements were made after incubation for 14 days at 21°C. and again after each of the 2 subsequent subcultures.

Results — There was a very great difference in the utilization of carbohydrates between cultures of *Ps. putrefaciens*. The only carbohydrate used by all cultures of the organism was glucose. Following glucose in numerical order of utilization were maltose (9), sucrose (9), dextrin (9), soluble starch (9), cellobiose (7), l-arabinose (6), glycogen (3), fructose (2), and inulin (1). Hence, this species of organism can not be identified by its action on carbohydrates. Of interest was the fact that none of the aliphatic alcohols, i.e., adonitol, dulcitol, erythritol, glycerol, d-mannitol, d-sorbitol, and inositol, were used.

3. Stimulation of Growth by B-Complex Vitamins

An investigation of the stimulation of growth of 10 cultures of *Ps. putrefaciens* by 9 different B-complex vitamins was made. The vitamins and their concentrations per 10 ml.

of basal medium were: Thiamine — 2 micrograms, riboflavin — 2 micrograms, pyridoxine — 2 micrograms, pantothenic acid — 2 micrograms, nicotinic acid — 2 micrograms, folic acid — 0.05 microgram, biotin — 0.02 microgram, para-amino-benzoic acid — 0.1 microgram, and choline — 5 micrograms. The basal medium was a chemically defined liquid composed of asparagine, l-glutamic acid, sodium citrate, gluconic acid, sodium ammonium phosphate, magnesium sulfate, and dipotassium phosphate·$3H_2O$.

Studies were made measuring the growth of the organisms in the presence of single vitamins, and in combinations of 8 of the 9 vitamins. Positive control tubes containing the 9 vitamins and vitamin-free negative control tubes were employed. Turbidimetric measurements were made after incubation for 5 days at 21°C. and again after each of the 2 subsequent transfers.

Results — Within the test conditions, the 9 B-complex vitamins either collectively or individually did not stimulate growth.

Lipolysis of Butterfat

The hydrolysis of natural butterfat by *Ps. putrefaciens* was measured by titration of the liberated fatty acids with a 0.05N alcoholic solution of potassium hydroxide.

Test tubes containing 25 mls. of sterile 35 per cent cream were inoculated with litmus milk cultures of each of the 10 representatives of the species *Ps. putrefaciens.* Daily, through a period of 6 days, 1 tube of each culture was removed from the 21°C. incubator; the cultured cream was churned and the resulting butter was washed and then melted in a hot water bath. The butter oil was centrifuged to facilitate the removal of water. It was filtered through Whatman No. 1 filter paper, weighed, titrated, and the values expressed as the ml. of 0.05N alcoholic potassium hydroxide required to neutralize 1 gram of the oil. A measurement of lipolysis was made again after 12 days had elapsed.

Results — The highest value obtained after a six-day incubation period was 0.22 ml. of 0.05N alcoholic potassium hydroxide as compared with an uninoculated control value of 0.09 ml. The arithmetic mean of the 10 cultures at the end of the six-day period was 0.149 ml. After a twelve-day incubation period, the arithmetic mean was 0.261 ml., with an individual extreme of 0.39 ml. of 0.05N alcoholic potassium hydroxide being required to neutralize 1 gram of the extracted butter oil. The negative control value at 12 days remained at 0.09 ml.

Although a definite upward trend in titration values existed, these values are very small when compared with the results of the lipolytic activity of such organisms as *Psuedomonas fragi* or *Achromobacter lipolyticum.*

Combination Studies

The most readily utilized substances were selected for use in the combination study. By comparison on a molecular weight basis, it was determined that l-malic, lactic, and fumaric acids afforded a greater growth response than any of the other nonnitrogenous carbon compounds studied. Hence, solutions of protolysate, casitone, and casamino acids at levels of 0.1, 1.0, and 2.5 per cent were combined with l-malic, lactic, and fumaric acids at levels of 0.1, 0.25, 0.5, and 1.0 per cent in 108 different combinations. The basal medium contained the same constituents as Koser's Citrate Medium with the exception of sodium citrate; dipotassium phosphate·$3H_2O$ was substituted for monopotassium phosphate. These 108 media were tested by use of 5 different cultures of the organism. Turbidimetric measurements were made after incubation for 5 days at 21°C. and again after 1 subtransfer. After determination of the combination best suited for optimum growth, a plating medium of the same composition was made. Equal amounts of the same dilution of 10 different cultures of *Ps. putrefaciens* were plated with Long's Agar and the experimental medium. This process was repeated with a plating medium of the same composition but lacking

the magnesium sulfate and sodium ammonium phosphate of the basal medium.

Results — The number of colonies developing on the first experimental medium were significantly higher than those on Long's Agar in 4 of 10 cultures. There was no difference in colony counts obtained from the remaining 6 cultures. Colony size was similar in all comparisons. The reddish pigmentation was slightly more pronounced on the experimental plating medium. The number of colonies developing on the second experimental medium were significantly higher than those on Long's Agar in 6 of 10 cultures. There was no difference in colony counts obtained from 3 of the other cultures. However, the number of colonies developing from the tenth culture was significantly higher on Long's Agar than on the experimental medium. There was no difference in colony size although pigmentation was enhanced on the experimental medium.

Summary

An investigation of the nutrition of *Ps. putrefaciens* was made. To define the growth requirements of this organism, growth responses to amino acids, other nitrogenous compounds, aliphatic monohydric alcohols, organic acids, carbohydrates, and B-complex vitamins were determined. The lipolysis of butterfat was also studied.

A plating medium suitable for the cultivation of this organism was developed. This plating medium was of the following composition:

Casitone (Difco)	2.5 per cent
l-Malic acid	1.0 per cent
Dipotassium phosphate·$3H_2O$	0.1 per cent
Agar	1.5 per cent
Adjust with sodium hydroxide to pH 7.0 ± 0.2	
Distilled water	to volume

VITA

George W. Reinbold, Jr., was born at Williamsport, Pennsylvania, on April 10, 1919. He attended elementary school at Williamsport, Pennsylvania, and at Merchantville, New Jersey. Secondary school was attended at Merchantville, New Jersey, at Media, Pennsylvania, and at Lansdowne, Pennsylvania, where he was graduated in 1936. In January 1942, he received a B.S. degree in Dairy Manufacturing from Pennsylvania State College.

Inducted into the Army in May 1942, he completed his basic training with the 8th Armored Division, attended the Chemical Warfare Service Officer's Candidate School, and was then assigned to the Army Air Force Chemical Warfare Service. He served with the 752nd Chemical Depot Company, Aviation, as Commanding Officer for approximately thirty-seven months and attained the rank of Major.

The author then attended the University of Illinois and in August 1947, was granted the M.S. degree in Dairy Manufactures.

Since March 1946, he has held a half-time assistantship in the Department of Dairy Science and during this time has been engaged in both research and instruction.